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## Using knockout mutants to reveal the growth costs of defensive traits

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**Abstract:** We used a selection of *Arabidopsis thaliana* mutants with knockouts in defence genes to demonstrate growth costs of trichome development and glucosinolate production. Four of the seven defence mutants had significantly higher size-standardised growth rates (SGR) than the wildtype in early life, although this benefit declined as plants grew larger. SGR is known to be a good predictor of success under high-density conditions, and we confirmed that mutants with higher growth rates had a large advantage when grown in competition. Despite the lack of differences in flowering-time genes, the mutants differed in flowering time, a trait strongly correlated with early growth rate. Aphid herbivory decreased plant growth rate and increased flowering time, and aphid population growth rate was closely coupled to the growth rate of the host plant. Small differences in early SGR thus had cascading effects on both flowering time and herbivore populations.

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# Using knockout mutants to reveal the growth costs of defensive traits

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We used a selection of *Arabidopsis thaliana* mutants with knockouts in defence genes to demonstrate growth costs of trichome development and glucosinolate production. Four of the seven defence mutants had significantly higher size-standardised growth rates (SGR) than the wildtype in early life, although this benefit declined as plants grew larger. SGR is known to be a good predictor of success under high-density conditions, and we confirmed that mutants with higher growth rates had a large advantage when grown in competition. Despite the lack of differences in flowering-time genes, the mutants differed in flowering time, a trait strongly correlated with early growth rate. Aphid herbivory decreased plant growth rate and increased flowering time, and aphid population growth rate was closely coupled to the growth rate of the host plant. Small differences in early SGR thus had cascading effects on both flowering time and herbivore populations.

**Keywords:** herbivore defence, Size-standardised growth rate, glucosinolates, trichomes, *Arabidopsis*.

## 1. INTRODUCTION

Plants deter herbivores through physical structures such as spines, thorns and hairs that reduce damage to leaf tissue [1, 2] and by producing toxic chemical compounds that reduce the growth rate or reproductive output of their enemies [3]. Such defences are assumed to be costly as they divert the plant's resources away from growth and reproduction [4-6]. However, experimental studies addressing fitness/defence trade-offs frequently fail to find the expected negative correlations [7-10], raising the question of whether such trade-offs are absent in many organisms (possibly through mechanisms which alleviate costs while maintaining resistance), or whether the methods employed to find them are inadequate [11].

*Arabidopsis thaliana* (L.) is attacked by a variety of pathogens [12] and herbivores, which include leaf-chewing caterpillars, sap-sucking aphids, flea beetles and leaf miners [13, 14]. As defence against these herbivores, *Arabidopsis* produces leaf hairs, called trichomes, and glucosinolates, a group of secondary metabolites [13]. Glucosinolate compounds are

produced by all species of the Brassicaceae [15] and plants show large variation for this trait in the field [16], most likely as a consequence of differential selection by herbivore communities [17]. The majority of glucosinolates either have aliphatic or indolic side-chains [18]. Both types of glucosinolates negatively affect generalist leaf-chewing herbivores while aliphatic glucosinolates tend to affect these herbivores more severely [19-22]. Phloem-feeding aphids are mainly impaired by indolic glucosinolates [23] although there is evidence from field studies that some aphid species are also impaired by aliphatic glucosinolates [24]. Previously, we demonstrated that the production of glucosinolate compounds appeared to be costly to the plant, as there was a negative correlation between plant growth rate and glucosinolate content [11]. We also showed that slow-growing plants suffered reduced herbivore damage. While suggestive, these correlations are not proof of causal relationships. Instead, the costs of defensive traits can be more directly estimated using knockout mutants, in which defence genes are disabled artificially. Ideally, knockout mutants only differ from the wildtype in target genes, and if mutant phenotypes are not more extreme than the phenotypes of naturally-

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occurring variants, we believe that such mutants can be used to address ecological questions.

In this study we compared the growth rate of mutants reduced in specific defence mechanisms with the wildtype. We conducted a multiple-harvest experiment and calculated size-standardised relative growth rates (SGR), for a range of plant sizes [see also 11, 25]. A reduction in early growth rate is a likely consequence of diverting resources to defence; however, it is possible that for isolated plants growing with no competition there will be no measurable reduction in final seed output. This could occur because the resources diverted to defence compounds early in life can be later reclaimed and redirected to the seeds. However, under competitive conditions, a reduction in growth rate is likely to have severe fitness costs; for example, Fakheran *et al.* [26] showed that early growth rate was a very good predictor of success when a mixture of *Arabidopsis* genotypes were grown under high-density, competitive conditions. However, when grown alone, these same genotypes did not differ in their final biomass [11].

In this study, we compared the growth rates of nine mutants with the wildtype in the presence and absence of the generalist aphid *Myzus persicae*. We also compared the growth rate of the aphid population on each of the ten genotypes and related this to the plant growth rate. Finally, we grew a subset of the genotypes in competition to test whether differences in early growth rates had greater fitness consequences under competitive conditions.

## 2. MATERIALS AND METHODS

### (a) Knockout mutants

We used knockout mutants created in the genetic background of the *Arabidopsis* accession Columbia (Col-0, see Table S1 for a description of mutant phenotypes). One mutant (*gll-2*) was originally created by x-ray mutagenesis and is deficient in trichome formation: the early leaves are entirely glabrous and there is greatly reduced trichome density on later leaves compared with the wildtype [27]. The *gll-2* mutant also shows decreased phenolic defence expression (Daniel J. Kliebenstein, unpublished data). All other mutants were originally created by T-DNA insertion. The mutants *myb28*, *myb29* and *myb28myb29* contain knockouts in transcription factors that decrease expression of aliphatic glucosinolates [21, 28] and the genotypes *cyp79B2*, *cyp79B3* and *cyp79B2cyp79B3* contain enzyme knockouts that decrease or abolish the indolic glucosinolate and camalexin pathways [29]. The genes *MYB28/MYB29* and *CYP79B2/CYP79B3* are tandem duplicated genes within their respective cellular pathway and are traditionally considered redundant [28, 29]. To control for non-target effects of transgenic plants such as the cost of expression of selection

marker genes, we included two genotypes with knockouts in genes not associated with defence and with no predicted fitness costs: *ppi1-2* and *nst1-2* [30, 31].

Even though all mutants used here were originally created by artificial gene knockout, similar phenotypes can be found in natural accessions of *Arabidopsis*. For example, the accessions est-0 (NASC 1148) and wil-3 (NASC 1598) are both completely glabrous, and glucosinolate levels vary considerably among natural accessions [32].

### (b) Experimental design

Plants were grown in a mixture of peat-based substrate (PP7, Tref Group, The Netherlands) and sand in a ratio 1:1. Each pot (diameter = 40 mm, depth = 70 mm) was sown with five seeds and cold stratified at 4° C for 48 hours. The pots were then moved to a glasshouse with supplemental artificial light at a 16h light / 8h dark cycle and 26° C day / 22° C night temperature. Plants were watered twice a week throughout the experiment and no additional nutrients were supplied. Five days after sowing, seedlings were thinned to leave only the most central seedling. Bolting (initiation of the flowering stem) was recorded for each plant to the nearest day. Six plants per genotype were harvested on days 5, 9, 13, 18, 23, 29 and 35 after germination. On day 5, the herbivore treatment was initiated by placing a single 1<sup>st</sup> instar aphid onto half of the remaining plants. The offspring of the introduced aphids (F<sub>1</sub>) were counted and removed at each harvest to keep herbivore pressure roughly constant among plant genotypes.

### (c) Size-standardised RGR of plants

We fitted an asymptotic regression model  $\log(\text{aboveground biomass})$  through time:

$$\log(M_{i,t}) = A_i + (\log(M_{i,0}) - A_i) \exp(-\exp(r_i)t) \quad (2.1)$$

where  $M_{i,0}$  is the starting mass of genotype  $i$  at  $t = 0$ ,  $A_i$  is the asymptotic mass as  $t \rightarrow \infty$  and  $r_i$  is the logarithm of the rate constant. The model was fitted with the function *gnls* in R [33] with genotype treated as a fixed effect. Models were compared based on their AIC values and size-standardised growth rates (SGR) were calculated with parameters taken from the best model. SGR is given by

$$SGR_i = \exp(r_i)(A_i - \log(M_{ref})) \quad (2.2)$$

where  $M_{ref}$  is a reference mass (for derivation of equation 2.2 see Appendix S1 and Rose *et al.* 2009).

### (d) Prediction intervals on SGR

*Gnls* produces point estimates and confidence intervals for the two estimated model parameters, the rate constant  $r_i$  and the asymptotic mass  $A_i$ . To estimate

confidence intervals for SGR (a function of these two parameters), we generated population prediction intervals [34, 35]. The method assumes that the distribution of the parameters is multivariate normal with a variance-covariance matrix given by the inverse of the information matrix. We used the function *mvrnom*, which selects multivariate normal random deviates, and the variance-covariance matrix given by the function *vcov*. We generated 1000 sets of parameters to calculate a distribution of differences between wildtype and mutant SGRs. The lower and upper 95% quantile of these distributions are the boundaries of the prediction intervals. Mutant SGRs are significantly different from wildtype SGR if the prediction interval does not include zero. Point estimates of SGR and prediction intervals were calculated at two reference masses ( $M_{ref}$  equation 2.2): an early SGR using the average mass at age = 5 days and a late SGR using the average mass at age = 29 days.

#### (e) Aphid rate of reproduction

Aphid performance was analysed by fitting the same asymptotic model (equation 2.1) to the log-transformed cumulative number of  $F_1$  aphids, thus generating a size-standardised relative growth rate of the aphid population. Estimates and prediction intervals of aphid SGR were calculated at two reference population sizes: 2 and 42 individuals, roughly corresponding to average offspring number on day 13 and 29.

#### (f) Early growth rate and competition

To determine whether differences in early growth rate affected the outcome of competition, we carried out a competition experiment with a subset of genotypes: *myb28*, *myb29* and the wildtype. Plants were grown in 5 x 5.5 cm pots filled with germination soil and maintained under long day (16h light / 8h dark) conditions in a controlled environment growth chamber. Prior to sowing, seeds were imbibed and cold stratified at 4° C for 3 days. In each pot, nine seeds were arranged into a square with an area of 1 cm<sup>2</sup>, thus closely surrounding the central seed with eight neighbours. Mutant central seeds were either surrounded by their own genotype or by the wildtype, while wildtype central seeds were surrounded by *myb28*, *myb29* or wildtype, resulting in a total of seven combinations. Each combination was replicated 12 times, half of which were harvested after three weeks and half after four weeks. There was some germination failure and only pots with more than 5 neighbour plants were kept, thus the sample size was decreased to 31 pots in week 3 and 28 pots in week 4. At day 18 for week 3 and day 25 for week 4, the rosette diameter of the central plant and two neighbours was recorded. Three days later, the same

plants were harvested and fresh weight was measured. Fresh weight or rosette diameter were analysed as a function of target genotype, neighbour genotype and harvest week using linear models.

### 3. RESULTS

#### (a) SGR of plant genotypes

The final asymptotic regression model included effects of plant genotype and herbivory on the rate constant  $r_i$  and the asymptotic mass  $A_i$  as judged by comparing AIC values (Table S2, Figure S1). There was no herbivory × plant genotype interaction. For the following analysis, only results from the control (without aphids) are shown.

Six of the seven defence mutants had significantly higher values of the rate constant  $r_i$  than wildtype, while the two mutants with knockouts in other genes did not differ from wildtype (Table 1). In contrast, all mutants had lower values of the asymptotic mass  $A_i$  compared to wildtype (Table S2). Early SGR was significantly higher than wildtype for the glabrous mutant *g11-2*, the indole glucosinolate mutants *cyp79B3* and *cyp79B2cyp79B3* and the aliphatic glucosinolate mutant *myb28* (Figure 1a). In later life, mutants tended to have equal or lower SGRs than the wildtype (Figure 1b).

As an unexpected result, we found that across the ten genotypes, early SGR is an excellent predictor of mean bolting age ( $r = -0.813$ ;  $F_{1,8} = 15.63$ ,  $p = 0.004$ ), i.e., fast-growing genotypes flowered earlier. This demonstrates that changes in early growth rate can influence flowering time, despite the fact that the mutant genotypes in question did not contain altered flowering genes. This apparently direct link between early growth rate and flowering time is confirmed by the aphid treatment: aphid feeding also decreased growth rate but increased bolting age in all genotypes (Table 1).

#### (b) Aphid rate of reproductive output

The asymptotic regression model included effects of plant genotype on the rate constant  $r_i$  and the asymptotic mass  $A_i$  (Figure S2, Table S3). With the exceptions of *ppi1-2* and *nst1-2*, none of the aphid SGRs calculated from this model were significantly different from wildtype (Figure 1c, d). However, the aphid rate of reproductive output on the different plant genotypes was strongly correlated with plant SGR at early stages ( $r = 0.877$ ,  $F_{1,8} = 26.67$ ,  $p = 0.0009$ ), and this correlation, even though weakened, was still present at the end of the experiment ( $r = 0.630$ ,  $F_{1,8} = 5.26$ ,  $p = 0.051$ ). Thus, aphid populations performed better on fast-growing genotypes.

#### (c) SGR and competition

Based on measurements of early SGR, we would predict that *myb28* should outcompete the wildtype,

Table 1. Parameters from the asymptotic regression model and bolting age of plant genotypes. Parameters for the wildtype are absolute values while the parameter values of mutants are differences from the wildtype. Bolting ages are absolute values. Aphid gives the overall difference in parameter values or age at bolting in the presence of aphids. Significant differences are in boldface.

Plant genotype	rate constant ( $r_i$ )	asymptotic mass ( $A_i$ )	bolting age
Wildtype	-2.25	3.58	18.6
gl1-2	<b>+0.12</b>	-0.27	<b>17.7</b>
cyp79B2	-0.00	-0.26	<b>19.8</b>
cyp79B3	<b>+0.18</b>	<b>-0.30</b>	18.0
cyp79B2cyp79B3	<b>+0.16</b>	<b>-0.36</b>	<b>16.4</b>
myb28	<b>+0.13</b>	-0.29	17.8
myb29	<b>+0.10</b>	<b>-0.37</b>	<b>17.3</b>
myb28myb29	<b>+0.12</b>	<b>-0.64</b>	<b>20.1</b>
nst1-2	+0.06	<b>-0.71</b>	<b>19.6</b>
ppi1-2	-0.04	-0.21	<b>22.2</b>
Aphid	-0.06	-0.14	<b>+0.47</b>

whereas *myb29* and wildtype should be equal competitors. In the analysis of fresh weight, neighbour genotype had a significant effect on the target genotype ( $F_{2,23} = 5.74$ ,  $p = 0.010$ ). In week 4, *myb28* target plants weighed  $0.18 (\pm 0.06, 1\text{SE})$  grams when surrounded by other *myb28* plants, but weighed  $0.41 (\pm 0.07, 1\text{SE})$  grams when surrounded by wildtype plants. Wildtype plants surrounded by wildtype neighbours weighed on average  $0.29 (\pm 0.07, 1\text{SE})$  grams, while wildtype plants surrounded by *myb28* neighbours weighed only  $0.09 (\pm 0.07, 1\text{SE})$  grams. The weight of *myb29* was not significantly affected by neighbour identity. The direction of the effects in week 3 and for rosette diameter in both weeks was similar but non-significant. Thus, it seems that the observed significant difference in early growth rate between *myb28* and wildtype has fitness consequences when the plants are grown in competition.

#### 4. DISCUSSION

Six of the seven genotypes with knockouts in defence genes had a higher rate constant ( $r_i$ ) than the wildtype but the asymptotic mass ( $A_i$ ) was lower for all mutants. As SGR is a function of both parameters, this meant that only four defence mutants had significantly higher early growth rate than the wildtype, and this difference decreased with increasing plant size. The observed differences in early growth rate were relatively small, but these differences had large effects on target plant size when growing in competition. For example, *myb28* has a higher initial growth rate than wildtype and thus should be able to outcompete it when the two genotypes are grown together. In support of this, *myb28* was more than twice as large with wildtype as with *myb28* neighbours and similarly, wildtype

individuals were larger with wildtype than with *myb28* neighbours. In contrast, the early growth rate of *myb29* (which was only grown with either wildtype or *myb29* neighbours in the competition experiment) is similar to wildtype and it was unaffected by neighbour identity when grown under competition. The large advantage observed under competitive conditions is not unexpected under scramble competition for resources, as a difference in early growth rate will lead to unequal resource uptake, and with a finite pool of resources, the plant with the higher uptake rate will gain a greater share of the total. In a recent study, Fakheran *et al.* [26] also showed that early growth rate was the best predictor of success in high-density competitive landscapes. Differences in growth rates among genotypes are thus also likely to be the underlying mechanism creating the sometimes ambiguous results from studies looking at kinship effects on competitive ability of plants [e.g. 36, 37].

Early growth rate was also a very good predictor of flowering time, a trait that varied by several days among genotypes, despite identical flowering genes. Aphid herbivory also reduced early growth rate and increased flowering time, again indicating a possible causal link between early growth rate and the decision to flower. Small differences in early growth rate are therefore biologically relevant, leading to a disadvantage in competition and to delayed flowering. Hence the production of defensive traits, and the consequent reduction in growth rate are likely to be costly to the plant. This supports findings from field experiments which show that both trichomes and glucosinolates have a visible fitness cost if herbivores are eliminated [e.g. 13]. It also supports theoretical work that assumes such a trade-off between defence and fitness.

Surprisingly, genotypes with knockouts in the homologous gene pairs MYB28/MYB29 and CYP79B2/CYP79B3 had relatively large differences in their growth rate. *cyp79B2* grew more slowly than *cyp79B3* and the double mutant, and *myb28* grew faster than *myb29* and the double *myb28/myb29* mutant. MYB28 and MYB29 are not completely functionally redundant and there is evidence of an incoherent feed-forward loop involving these two genes that complicates our ability to place them in a linear pathway [38]. Likewise, CYP79B2 and CYP79B3 are not completely functionally redundant, with the genes having quantitative preferences to the camalexin versus indole glucosinolate pathways. How the fluxes are reshuffled in the single mutants is not currently understood and as such, the double CYP79B2B3 is a cleaner background to directly interpret [39]. These data suggest that the genes MYB28/MYB29 and CYP79B2/CYP79B3 are involved in non-linear pathways that are not completely understood and will require further

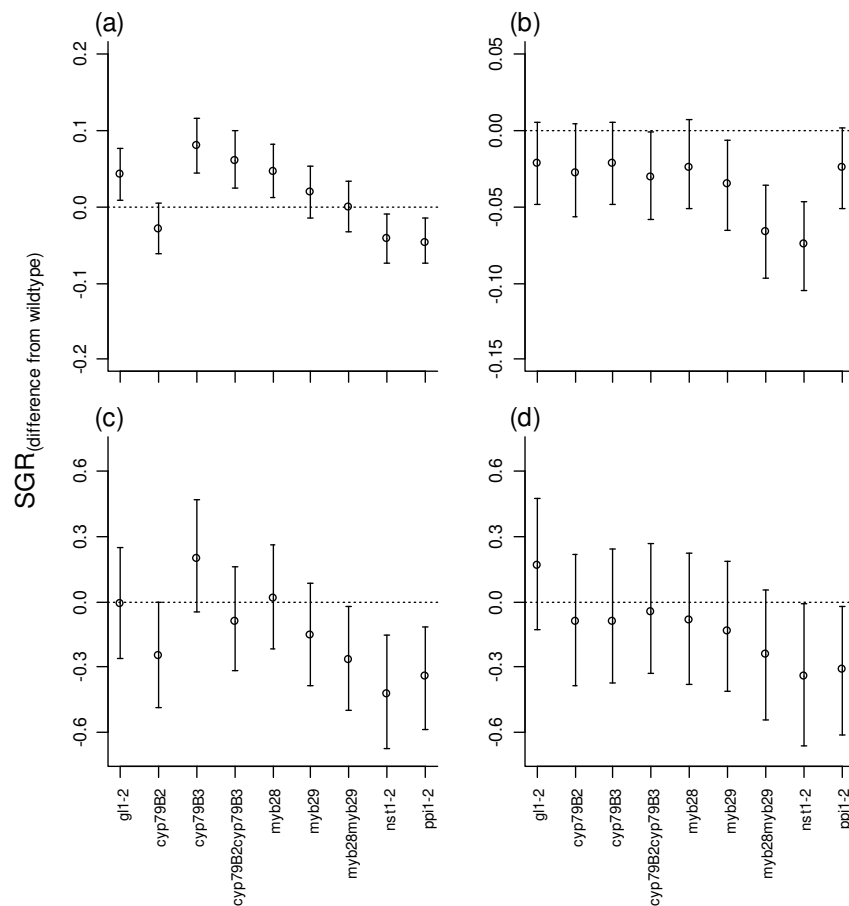


Figure 1. Differences in size-standardized relative growth rates (SGRs) of mutant plants from wild-type in (a,b) and (c,d) population SGRs of aphids feeding on mutant plants. For plants, early SGR is calculated for average mass (a) at age = 5 days and (b) at age = 29 days, while for aphids, SGR is calculated at the average population size (c) when plant age = 13 days and (d) when plant age = 29 days. Dotted lines represent zero difference from wild-type in SGR, error bars show 95% prediction intervals.

research to parse. This does suggest that single gene mutants in any background may be more complicated to interpret than is traditionally considered.

Defence mutants benefited from the lack of defensive traits in early life; but, as plants grew larger, this benefit apparently disappeared. In contrast, the two mutants with knockouts in other (non-defence-related) genes performed worse than the wildtype at all sizes – a phenomenon that was not observed previously; hence these mutants were thought to be neutral [30, 31]. The poor performance of the two non-defence-related mutants in our study may be due to the growing conditions: our plants were grown in small pots in a sand/soil mixture with no additional nutrients, and this could be a more stressful environment than that normally used for genetic work. That all mutants had poorer performance at larger sizes is possibly due to pleiotropic effects, as disabling a gene usually affects several functions. It could also be due to the expression of selection marker genes, which might have associated costs (although this would not explain the poor performance of *gl1-2*, which is not a transgenic).

According to optimal defence theory [40] plants should follow different defence strategies before and after bolting, hence the decline in mutant SGRs with

respect to wildtype could also represent a change in the value of defensive traits. Prior to bolting, growth is mass dependent and removal of leaf tissue by herbivores should be particularly costly, thus plants should invest heavily in leaf defences. Mutant plants, unable to produce such defensive traits, then have additional resources available for growth. After bolting, the inflorescence becomes the most valuable plant organ. However, at least part of the defensive compounds in the inflorescence are relocated from rosette leaves [18]; wildtype plants might thus synthesise less glucosinolates *de-novo* during the post-flowering period, hence decreasing the relative advantage of knockout mutants.

All plant genotypes were similarly susceptible to aphid herbivory and aphid performance was not generally better on genotypes with knockouts in defence genes. However, if aphids remove a constant fraction of the plant's resources, we still expect faster-growing plants to support higher aphid population growth (see Hautier *et al.* [41] for a similar situation with a parasitic plant, *Rhinanthus alectorolophus*). This was indeed the case, as aphid population growth rate was strongly correlated with plant SGR. The relatively small differences in aphid population size on wildtype and mutant plants in our study is probably

partly a result of keeping aphid densities low by constantly removing offspring. Low herbivore densities might in turn be unable to trigger a full defensive response by the plants; as part of the defence response of *Arabidopsis* is only induced by herbivore feeding [23, 42, 43]. That high concentrations of certain glucosinolate compounds can affect aphid feeding has been shown by Kim & Jander [23], who demonstrated that indolic, but not aliphatic glucosinolates deterred *M. persicae* when applied in artificial diets. However, Kim *et al.* [44], too, failed to show increased aphid reproduction on the *cyp79B2cyp79B3* double-knockout mutant and only demonstrated decreased reproduction on a mutant overexpressing indolic glucosinolates. The specific mechanism involved in plant defence against aphids thus remains unclear, while the relevance of glucosinolates in defence against leaf-chewing herbivores has been demonstrated repeatedly [19–22].

In summary, mutants with knockouts in defence genes generally grew faster at small sizes than the wildtype. This enhanced early growth rate gave them an advantage in competition and allowed them to flower earlier. Combined with earlier work demonstrating a negative correlation between glucosinolate concentrations and growth rates, this study supports the hypothesis that the defence traits of *Arabidopsis* are costly to the plant. While knockout mutants helped to reveal these costs, such mutants can exhibit growth disadvantages, particularly in later life, and especially when grown under nutrient-poor conditions, and hence should be used with caution.

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